(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 April 2002 (04.04.2002)

PCT

(10) International Publication Number
WO 02/27017 A2

(51) International Patent Classification[7]: C12Q 1/00

(21) International Application Number: PCT/US01/30186

(22) International Filing Date: 27 September 2001 (27.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/235,620 27 September 2000 (27.09.2000) US

(71) Applicant: **BIOSOURCE INTERNATIONAL** [US/US]; 820 Flynn Road, Camarillo, CA 93012 (US).

(72) Inventors: **REAGAN, Kevin, J.**; 5833 Middle Crest Dr., Agoura Hills, CA 91301 (US). **SCHAEFFER, Erik**; 12 Greystone Lane, Hopkinton, MA 01748 (US). **WANG, Jimin**; 657 Camino Del Mar, Thousand Oaks, CA 91320 (US).

(74) Agent: **MCDONNELL, John, J.**; McDonnell Boehnen Hulbert & Berghoff, 300 South Wacker Drive, Chicago, IL 60606 (US).

(81) Designated States *(national)*: CA, JP.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/27017 A2

(54) Title: METHOD FOR QUANTIFYING PHOSPHOKINASE ACTIVITY ON PROTEINS

(57) Abstract: The invention involves a method for measuring phosphorylation of proteins and, as such, is an indicator of protein kinase activity. The method involves the *in vitro* phosphorylation of a target protein but subjecting that protein (non-phosphorylated) to reaction mixture containing all reagents, including phosphokinase which allow the creation of a phosphorylated form of protein. The phostphorylated protein is measured by contacting it with an antibody specific for the phosphorylation sites(s). The invention includes antibodies useful in practicing the methods of the invention. The invention particularly relates to phosphorylation of Tau, Rb and EGFR proteins and antibodies specific for the site of phosphorylation of the Tau, Rb or EGFR proteins.

METHOD FOR QUANTIFYING PHOSPHOKINASE ACTIVITY ON PROTEINS

BACKGROUND OF THE INVENTION

This application claims the priority of provisional application No. 60/235,620 filed on September 27, 2000.

FIELD OF THE INVENTION

This invention relates to assays and reagents for measuring protein kinase activity *in vitro*.

BACKGROUD OF THE ART

Drug development efforts involve a continuum of activities initiated by target selection of a molecule. Since all drugs work at the level of the cell, those targets are usually proteins that somehow are involved in cellular communication pathways. Signal transduction pathways are key to normal cell function. Aberrations in the expression of intracellular molecules and coordinated interactions of signal transduction pathways are associated with a variety of diseases and, thus, are the focus of drug discovery efforts. Phosphorylation of proteins in signal transduction pathways is one of the key covalent modifications that occur in multicellular organisms. The enzymes that carry out this modification are the protein kinases, which catalyze the transfer of the phosphate from ATP to tyrosine, serine or threonine residues on protein substrates. Phosphorylation of these amino acid residues modulates enzymatic activity and creates binding sites for the recruitment of downstream signaling proteins. Because protein kinases are critical components of cellular signaling pathways, their catalytic activity is strictly regulated. Abnormalities in protein kinase activity alter patterns of phosphorylation with subsequent affects on the cell function. Many drug discovery efforts involve the identification of chemicals that selectively suppress protein kinase activity. This invention is designed to provide assays and reagents to monitor protein kinase activity.

The targeted residues for phosphorylation can be contained in a full-length, biologically active molecule of recombinant or natural origin. Most methods currently employed for measuring protein kinase activity use peptide substrates, which include the targeted phosphorylation residue. This art is taught in US 6, 066,462 (Quantitation of individual protein kinase activity) incorporated herein by reference. This method differs from the present invention in that the peptide substrate does not contain all possible phosphorylation sites that can be acted on by kinases and thus may not truly reflect activity on a natural protein. The invention described herein can be used with whole molecule or fragments, of natural or recombinant origin. Also, the delineation of activity at different residue sites requires in the invention, a different PSSA for detection as opposed to a different peptide in US 6,066,462.

Another method for detection of kinase activity involves use of a generic antibody that binds to all phospho-tyrosine residues. This method is described in US 5,766,863 (Kinase receptor activation assay) incorporated herein by reference. This method suffers from an inability to discriminate among phosphorylated tyrosine residues on a molecule. This method is also incapable of detecting phospho-serine or phospho-threonine molecules since the antibody does not detect such phosphorylated residues. In contrast, the method described herein uses antibodies, which bind to the sequence specific residues surrounding the phosphorylated amino acid plus the phospho-residue itself. The reagents used in this invention are capable of detecting phosphorylated threonine, serine or tyrosine molecules.

Three examples are employed; all of which involve important molecular targets of current interest in disease-oriented pharmaceutical study.

Currently, neurobiologists are focusing efforts on the proteins in the brain that can be associated with disease. One such protein is called Tau, a neuronal microtubule-associated protein found predominantly in axons. The function of Tau is to promote tubulin polymerization and stabilize microtubules, but it also serves to link certain signaling pathways to the cytoskeleton. Tau phosphorylation regulates both normal and pathological functions of this protein. Tau, in its hyper-phosphorylated form, is the

major component of paired helical filaments (PHF), the building block of neurofibrillary lesions in Alzheimer's disease (AD) brain. Hyperphosphorylation impairs the microtubule binding function of Tau, resulting in the destabilization of microtubules in AD brains, ultimately leading to neuronal degeneration. Hyperphosphorylated Tau is also found in a range of other central nervous system disorders. Numerous serine/threonine kinases, including GSK-3β, PKA, PKC, CDK5, MARK, JNK, p38MAPK and casein kinase II, can phosphorylate Tau.

Detection of *in vitro* kinase activity is critical for screening compounds that may be able to inhibit this activity and therefore could be useful in ameliorating various neurodegenerative diseases where Tau phosphorylation is abnormally high. Current efforts exist to identify drugs that might suppress kinase activity towards the Tau protein; however, these methods suffer from poor sensitivity and low specificity. Phosphorylation at individual Serine or Threonine residues within the Tau protein has been shown to correlate with disease. This invention overcomes both of these deficiencies in the described 'art'.

US Patent 5,601,985 relates to methods of detecting abnormally phosphorylated Tau Protein; US Patent 5,843,779 relates to monoclonal antibodies directed against the microtubule-associated protein, Tau, and hybridomas secreting these antibodies; US Patent 5,733,734 relates to methods of screening for Alzheimer's disease or disease associated with the accumulation of paired helical filaments and US Patent 6,066,462 relates to quantitation of individual protein kinase activity. These patents are incorporated herein by reference.

Other models exist to show the general applicability of this format for monitoring kinase activity. For the purposes of illustration, we have designed assays around an intranuclear molecule Retinoblastoma protein (Rb protein) important in cell cycle regulation and a cell surface receptor molecule (EGFR).

Retinoblastoma protein (Rb), the tumor suppressor product of the retinoblastoma susceptibility gene, is a 110 kDa protein which plays an important role in regulating cell

growth and differentiation. Loss of its function leads to uncontrolled cell growth and tumor development. Mutational inactivation of the Rb gene is found in all retinoblastomas and in a variety of other human malignancies including cancers of breast, lung, colon, prostate, osteosarcomas, soft tissue sarcomas, and leukemia. Central to the role of the Rb protein as a tumor suppressor is the ability of Rb to suppress inappropriate proliferation by arresting cells in the G1 phase of the cell cycle. Rb protein exerts its growth suppressive function by binding to transcription factors including E2F-1, PU.1, ATF-2, UBF, Elf-1, and c-Abl. The binding of Rb protein is governed by its phosphorylation state. Hypo- or under-phosphorylated Rb binds and sequesters transcription factors, most notably those of the E2F/DP family, inhibiting the transcription of genes required to traverse the G1 to S phase boundary. The cell cycle inhibitory function is abrogated when Rb undergoes phosphorylation catalyzed by the complex of cyclins/cyclin-dependent protein kinases (cdks).

Rb contains at least 16 consensus serine/threonine sequences for cdk phosphorylation, although the significance of all these sites is unclear. It has been demonstrated that phosphorylation of threonine 821 on Rb is catalyzed by cdk2/complex such as Cyclin E/cdk2 and Cyclin A/cdk2. The phosphorylation of threonine 821 disrupts the interaction of Rb with the proteins containing the sequence LXCXE. The dephosphorylation of Rb protein returns Rb to its active, growth suppressive state. Removal of phosphates on Rb appears to be carried out by a multimeric complex of protein phosphatase type 1 (PP1) and noncatalytic regulatory subunits at the completion of mitosis. The quantitation of Rb phosphorylated at specific amino acid residues gives important information regarding the activity of kinases as well as the functional state of activation of the Rb protein itself. For the purposes of illustration, we designed an assay to quantitate the amount of Rb protein that is specifically phosphorylated at threonine 821. This ELISA does not recognize Rb phosphorylated at sites other than [pT^{821}] or non-phosphorylated Rb. Samples can be controlled for Rb content by parallel measurement of total Rb.

WO 01/11367 (Assay of the phosphorylation activity of cyclin/CDK complex on retinoblastoma (RB) protein for identifying compounds which modify this activity) describes a method for detecting kinase activity by ELISA using a synthetic peptide and

a monoclonal antibody that recognizes the phosphorylated form of the peptide. The basis of this method is the coating of a solid phase with a synthetic peptide containing the consensus sequence of a region upon which a kinase acts. The peptide is allowed to come in contact with a kinase that allows a specific residue on that peptide to become phosphorylated. The activity of the kinase is reflected in the by the binding of the monoclonal antibody. Our invention differs from WO 01/11367 by the ability to use natural protein as the substrate for kinase activity. This feature is superior to the use of peptides since all naturally occurring phosphorylation sites would be present. The use of a single monoclonal antibody recognizing phosphoserine (clone 2B9) does not allow any discrimination of specific phosphorylation sites among those on the naturally occurring protein. Our use of specific PSSAs allows that distinction as well as the detection of phosphothreonine and phosphotyrosine residues.

In another example, a cell surface receptor was studied and a kinase-dependent assay designed. Epidermal growth factor receptor (EGFR) belongs to the family of receptor tyrosine kinases (RTKs), which regulate cell growth, survival, proliferation and differentiation. EGFR is expressed at full length as a 170 kDa type I transmembrane glycoprotein which consists of an extracellular ligand-binding domain, a single hydrophobic transmembrane region, and an intracellular, highly conserved region involved with signal transduction. Several deletions in the extra- and intracellular domain of the EGFR have been found in number of tumors. For example, EGFRvIII is a 145 kDa protein with a deletion of exons 2-7 in EGFR mRNA. A 100 kDa truncated EGFR without the cytoplasmic domain is observed in the culture supernatant from A431 cells, a human epidermal carcinoma cell line.

EGFR is activated by binding of a number of ligands such as EGF, transforming growth factor α (TGFα), amphiregulin, betacellulin, heparin binding EGF-like growth factor (HB-EGF) and epiregulin. The binding causes EGFR homo- and heterodimerization and rapid activation of its intrinsic tyrosine kinase followed by autophosphorylation of multiple tyrosine residues in the cytoplasmic domain. The phosphorylation of tyrosine residues in the COOH-terminal tail of the molecule serve as binding sites for cytosolic signaling proteins containing Src homology 2 (SH2) domains.

Several sites of *in vivo* phosphorylation have been identified in the EGFR including Tyr^{845}, Tyr^{992}, Tyr^{1068}, Tyr^{1086}, and Tyr^{1173}. These sites bind and activate a variety of downstream signaling protein which contain SH2 domains, including growth factor receptor-binding protein 2 (Grb2), Src homology and collagen domain protein (Shc) and phospholipase C-γ (PLCγ). The binding of these or other signaling proteins to the receptor and/or their phosphorylation results in transmission of subsequent signaling events that culminate in DNA synthesis and cell division.

Elevated expression and/or amplification of the EGFR have been found in breast, bladder, glioma, colon, lung, squamous cell, head and neck, ovarian, and pancreatic cancers. Selective compounds have been developed that target either the extracellular ligand-binding domain of EGFR or the intracellular tyrosine kinase region, resulting in interference with the signaling pathways that modulate mitogenic and other cancer-promoting responses. These potential anticancer agents include a number of small molecule, tyrosine kinase inhibitors.

SUMMARY OF THE INVENTION

The invention describes assays and reagents for quantitating phosphorylation of proteins. The method involves subjecting a protein to a phosphokinase that will phosphorylate the protein and contacting the phosphorylated protein with an antibody specific for the phosphorylated site and detecting the antibody bound to the phosphorylated site. The invention includes antibodies useful in practicing the methods of the invention. The invention particularly relates to phosphorylation of Tau, Rb, and EGFR proteins and antibodies specific for the site of phosphorylation of the Tau, Rb, and EGFR proteins.

In each example system, the targeted protein (Tau, Rb or EGFR) is phosphorylated *in vitro* or *in vivo* and the specific phosphorylation event is detected using a highly selective phosphorylation site-specific antibody (PSSA). The appearance or disappearance of the targeted phosphorylation event can be quantified as a percentage of total protein that may be phosphorylated at each site.

The highly specific nature of the PSSAs allows parallel independent measurement of multiple phosphorylation sites on one protein. Moreover, different kinases can be measured simultaneously by using different PSSAs that selectively target different sites in the protein, thereby providing an avenue for generating phosphorylation site profiles. In contrast to existing methods that quantitate phosphorylated proteins as a diagnostic or prognostic indication of disease, this invention measures protein kinase enzymatic activity that results in the phosphorylation of proteins at a specific site(s). This method is also amenable to large-scale 'High Throughput Screening' formats currently being used by pharmaceutical and biotech companies to discover new drugs.

The term antibody used herein refers to monoclonal, polyclonal, Fab and F(ab)2 fragments, genetically engineered antibodies or fragments thereof. Antibodies may be modified, for example, humanized to minimize cross species reaction. Pan antibodies as the term is used herein refers to antibodies that bind to phosphorylated and non-phosphorylated forms of the protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates specificity of the Anti-phospho Tau [pS^{199}] phosphorylation site-specific antibody (PSSA).

Figure 2 illustrates Anti-phospho Tau [pS^{214}] PSSA specificity.

Figure 3a and 3b illustrate detection of total Tau vs. Tau phosphorylated at the PKA/serine 214 site by ELISA.

Figure 4a-b illustrate detection of Tau phosphorylated at GSK-3β/serine 199/202 (4a) vs. total Tau (4b) sites by ELISA.

Figure 5 illustrates a dose-response curve generated in an ELISA using the Tau serine 214 PSSA.

Figure 6 illustrates the specificity of the Tau PSSAs in an ELISA to detect Tau phosphorylation catalyzed by PKA vs. GSK-3β enzymes.

Figure 7 illustrates that multiple GSK-3β phosphorylation sites on Tau can be detected by ELISA using Tau PSSAs.

Figure 8 illustrates that a specific inhibitor of PKA activity selectively inhibits the phosphorylation on serine 214 of Tau but does not interfere with GSK enzyme activity as demonstrated using Tau [pS^{214}] and Tau [pS^{199}] PSSAs as detected by ELISA.

Figure 9 defines the specificity of the anti-Rb [pT^{821}].

Figure 10 shows studies to determine the specificity of the Rb [pT^{821}] ELISA.

Figure 11 shows the specificity of the Rb [pT^{821}] ELISA for threonine 821 as determined by peptide competition.

Figure 12 shows the application of the Rb [pT^{821}] ELISA in evaluating kinase activity in Jurkat cells were grown in the presence of the kinase inhibitor, staurosporine.

Figure 13 illustrates the specificity of the EGFR PSSA [pY^{1173}].

Figure 14 shows the specificity of the EGFR [pY^{1173}] ELISA for tyrosine residue 1173 as determined by peptide competition.

Figure 15 demonstrates the response curve of phosphorylation of EGFR in A431 cells after treatment with EGF using the EGFR [pY^{1173}] ELISA.

Figure 16 shows the application of the EGFR [pY^{845}] ELISA in evaluating kinase activity in A431 cells were grown in the presence of the tyrosine kinase inhibitor, PD158780.

DETAILED DESCRIPTION OF THE INVENTION

Tau System: The Tau protein system demonstrates the utility of this invention on a protein that is found both intracellularly and extracellularly in normal and pathological conditions. The Tau protein has multiple phosphorylation sites acted upon by multiple protein kinases. Phosphoserine and phosphotyrosine residues exist. Both mono-phospho and dual-phosphoresidues are distinguishable in this model system.

Tau Recombinant Protein: Full length Tau-441 protein is purified recombinant protein derived through cloning of human Tau cDNA and expressed in *E.coli*. The protein is purified via standard methods. This protein is commercially available from multiple vendors.

Tau pS^{199} PSSA: Rabbits were immunized with a chemically synthesized and KLH conjugated phosphopeptide corresponding to the region of the longest isoform of the Tau protein that includes serine 199. The chemically synthesized phosphopeptides (RSGYS(**pS**)PGSPG) is sequence ID #1. The Tau pS^{199} PSSA was purified from rabbit serum by sequential epitope-specific chromatography. The antibody was negatively preadsorbed using a non-phosphopeptide corresponding to the site of phosphorylation to remove antibody that is reactive with non-phosphorylated Tau. The final product was generated by affinity chromatography using the peptide that is phosphorylated at serine 199. This antibody recognizes specifically the Tau protein when phosphorylated on serine 199, as demonstrated by peptide competition analysis in a western blotting assay. Serine 199 is phosphorylated *in vitro* and *in vivo* by glycogen synthase kinase-3β (GSK-3β). This reagent is commercially available.

PSSA Tau specificity is shown in figure 1. Cell extracts from African green monkey kidney (CV-1) cells, stably expressing human four repeat tau and a protein phosphatase inhibitor, were resolved by SDS-PAGE on a 10% Tris-glycine gel. The proteins were transferred to nitrocellulose. Membranes were incubated with 0.50 μg/mL anti-phosphoTau [pS^{199}], following prior incubation in the absence (a) or presence of the peptide immunogen (b), or the non-phosphopeptide corresponding to the tau phosphopeptide (c). After washing, membranes were incubated with goat $F(ab')_2$ anti-rabbit IgG alkaline phosphatase and

bands were detected using the Tropix WesternStar™ detection method. The data in Figure 1 show that only the phosphopeptide corresponding to this site blocks the antibody signal, illustrating the specificity of the anti-tau [pS^{199}] antibody for this phosphorylation site.

Tau [pS^{214}] PSSA. The procedures for generating this antibody were similar to those described above for the Tau pS^{199} PSSA. The chemically synthesized phosphopeptide was derived from the region of the longest isoform of Tau protein that includes serine 214 (GSRSRTP(**pS**)LPTPP) sequence ID#2. This antibody recognizes specifically the Tau protein when phosphorylated on serine 214, as demonstrated by peptide competition analysis in a western blotting assay. Serine 214 is phosphorylated in vitro and *in vivo* by cAMP-dependent protein kinase (PKA). This reagent is commercially available Biosource International.

Tau pS^{214} PSSA specificity is show in Figure 2. SF-9 cell extracts, expressing human four repeat tau, were resolved by SDS PAGE on a 10% Tris-glycine gel. The proteins were transferred to nitrocellulose. Membranes were incubated with 0.50 μg/mL anti-phospho tau [pS^{214}], following prior incubation in the absence (a) or presence of the peptide immunogen (b), or the non-phosphopeptide corresponding to the tau phosphopeptide (c). After washing, membranes were incubated with goat $F(ab')_2$ anti-rabbit IgG alkaline phosphatase and bands were detected using the Tropix WesternStar™ detection method. The data in Figure 2 show that only the phosphopeptide corresponding to this site blocks the antibody signal, illustrating the specificity of the anti-Tau [pS^{214}] antibody for this phosphorylation site.PSSAs to other Tau sites [pS^{202}, pS^{396}, pT^{181}, pS^{199}/pS^{202}, pS^{404}] are characterized using similar methods.

Pan-Tau polyclonal Antibody

Rabbits were immunized with the recombinant Tau protein. The antibody was purified from the rabbit serum using a protein-A affinity column. This antibody recognizes multiple antigenic sites on Tau protein. This antibody will bind to both non-phosphorylated and phosphorylated forms of Tau protein.

Tau-5 monoclonal Antibody (mAb)

The mouse mAb to Tau was raised using purified bovine microtubule-associated proteins (MAPs) as the immunogen. The resulting hybridoma was produced by fusing immunized BALB/c mouse splenocytes and mouse myeloma Sp2/0-Ag14 cells. It shows no cross-reaction with other MAPs or tubulin. It reacts with the non-phosphorylated as well as the phosphorylated forms of Tau and the reactive epitope maps to residues 210-230. This reagent is commercially available from Biosource International.

Total Tau ELISA and Phospho-Tau ELISA

A concentration of 2.5 μg/mL of Tau-5 monoclonal antibody in carbonate buffer, pH 9.4, was incubated at 100 μL/well in microtiter plates at 4°C overnight. The wells were washed with a PBS/Tween-20 solution three times followed by blocking on other sites on the plastic surface with a buffered solution containing unrelated proteins such as BSA for 2 hours at room temperature. GSK-3β phosphorylated Tau, PKA phosphorylated Tau, and non-phosphorylated Tau were added to the wells at various concentrations and incubated for 1 hour at room temperature. After three washings with Washing Buffer, the wells were incubated respectively with Tau pS^{214} PSSA, Tau pS^{199} PSSA and Pan-Tau antibodies at the optimized concentrations (ranging from 0.1 to 1 μg/mL) for 1 hour at room temperature. The plates then were washed three times with Washing Buffer, followed by the addition of an HRP conjugated anti-rabbit IgG secondary antibody at 1:5000 dilution for 1 hour at room temperature. After washing, 100 μL of Stabilized Chromogen was added to each well and then incubated for 20 minutes at room temperature in the dark. The O.D. values at 450 nm were measured following the addition of stop solution to each well.

Kinase Reactions

Phosphorylation of Tau using PKA occurred as follows. PKA was purchased from New England Biolabs. Recombinant Tau protein (1 μg) was incubated with various concentrations of PKA enzyme in buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 100 μM ATP for 1 hour at 30°C.

Phosphorylation of Tau using GSK-3β

GSK-3β was purchased from Upstate Biotechnology Inc. Recombinant Tau protein (1 μg) was incubated with various concentrations of the enzyme in buffer containing 40 mM

HEPES (pH 7.2), 5 mM $MgCl_2$, 5 mM EDTA, 100 μM ATP, and 50 μg/mL heparin for 1 hour at 30°C.

Figures 3a and 3b show the assessment of total Tau and selective Tau phosphorylation at the PKA/ Ser^{214} site by ELISA. In figure 3a, phosphorylated Tau was placed into an ELISA with detection either by PSSA specific for Tau pS^{214} or by pan-Tau antibody. Both detection antibodies detected the phosphorylated Tau with equal signals. In figure 3b, non-phosphorylated Tau was placed into the same assay. As expected, the Tau pS^{214} failed to detect the Tau protein without the phosphate group whereas the pan-Tau detector did quantitate the protein

Figures 4a and 4b show the assessment of total Tau and selective Tau phosphorylation at the GSK-3β/ $Ser^{199/202}$ sites by ELISA. Figure 4a uses either non-phosphorylated Tau or GSK-3β- phosphorylated Tau in the ELISA with a detector of Tau $pS^{199/202}$. Non-phosphorylated Tau does not react positively in the ELISA whereas the phosphorylated Tau shows strong results. If pan-Tau is used as the detector, both proteins are readily detected (figure 4b).

Figure 5 shows the direct relationship between the amount of phosphoTau detected by ELISA as a relationship of the quantity of kinase activity added to the *in vitro* reaction. Various amounts of PKA enzyme were used to phosphorylate the Tau protein. Starting with the highest concentration of PKA, 5 units, (PKA tau 1), the PKA enzyme was then serially diluted 1:2 as shown, followed by a 1:1000 dilution and then applied to each well of the ELISA assay. Detection of phosphoTau was performed using a PSSA to Tau that is phosphorylated at Ser^{214} (a PKA site). These data indicate that the lower the amount of kinase in the reaction, the lower the amount of phosphoprotein produced is detected in the ELISA. Thus, the ELISA is indirectly a measure of phosphokinase activity.

Figures 6a and 6b shows the specificity in detecting Tau protein phosphorylation catalyzed by PKA vs. GSK3β enzymes using the Tau PSSAs and ELISA. The results demonstrate that the Tau pS^{214} PSSA ELISA only detects Tau when phosphorylated by PKA and the Tau pS^{199} PSSA ELISA only detects Tau when phosphorylated by GSK3β.

Figure 7 shows that GSK3β enzyme can phosphorylate multiple sites on Tau protein and PSSAs can independently detect the phosphorylated sites at Tau pT^{181}, Tau pS^{202}, Tau pS^{199}/pS^{202}, Tau pS^{396}, and Tau pS^{404}. This provides evidence that the ELISA is useful in creating a profile of phosphorylation events on the protein subjected to kinase enzyme activity.

Figure 8 shows the specificity of kinase reaction when tested as a profile with two antibodies, one specific for a PKA phosphorylation site (pS^{214}) and the other for a GSK site (pS^{199}) on Tau protein. A PKA-specific inhibitor, PKI (heat-stable inhibitor of c-AMP-dependent protein kinase; New England Biolab), was mixed at various ratios of inhibitor to enzyme (either PKA or GSK) and the resultant mixture analyzed by ELISA using the Tau PSSAs. The PKA-specific inhibitor altered the kinase activity of the pS^{214} site alone. These data again attest to the specificity of the ELISA and the ability to independently monitor kinase activities on the same protein at different sites. These data also show the capability of screening for drug interference of kinase activity.

Antibodies to other tau sites shown in Table II are also representative of the invention. Som of the phosphorylated sites are known to be associated with disease as further indicated in Table II.

TABLE II

Phospho Site	Disease Linked (Y/N/?)	Notes (NGD = Neurodegenerative disease; FTD =)
T39	?	Phosphorylated by Casein kinase II
T181, hu	?	Involved in pretangle process?
S184	Y	Phosphorylated by GSK-3b; disrupts microtubule network
S195	Y	Phosphorylated by GSK-3b; disrupts microtubule network
S198	Y	Phosphorylated by GSK-3b; disrupts microtubule network
S199	Y	Phosphorylated by GSK-3b; linked to hereditary FTD
S202	Y	Microtubule-dependent phosphorylation by CDK 5 and GSK-3b; linked to hereditary NGD
T205	?	Microtubule-dependent phosphorylation by CDK 5 and GSK-3b
T212	Y	Specific for NGD processes; phosphorylated by GSK-3b and PKA
S214	Y	Specific for NGD processes; may block aggregation; phos'd by PKA
2T17	?	
T231	Y	Involved in pretangle process?; phos'd by GSK-3b and cdc2/CDK1
S235	?	Microtubule-independent phosphorylation by GSK-3b
S262	Y	May block aggregation; phosphorylated by CAM K II and GSK-3b; major site in AD brain
S320	?	
S324	?	
S356	Y	Involved in pretangle process?; AD pathway; major site in AD brain; phosphorylated by GSK-3b
S361	?	
S396	N	Phos'd by GSK-3b
S400	?	Phos'd by GSK-3b
T403	?	
S404	?	Involved in pretangle process?; microtubule-independent phosphorylation; phosphorylated by GSK-3b
S409	Y	AD pathway; phosphorylated by PKA
S412	?	AD pathway
S413	Y	AD pathway; phosphorylated by GSK-3b
S416	?	Phosphorylated by CAM K II
S422	Y	Linked with several NGD's; phosphorylated by MAPK

Rb System: This model system presents an intra-nuclear protein that is large with multiple phosphorylation sites acted upon by multiple protein kinases. Phosphoserine and phosphotyrosine residues exist. Both mono-phospho and dual- phosphoresidues are distinguishable in this model system.

Rb protein: Full length Rb protein is purified recombinant protein derived through cloning of human Rb cDNA and expressed in *E. coli.* The protein is purified via standard methods. This protein is commercially available from multiple vendors.

Rb [pT^{821}] PSSA: The rabbit antiserum was produced against a chemically synthesized phosphopeptide derived from a region of human Rb that contains threonine 821. Antibody was purified from rabbit serum by sequential epitope-specific chromatography. The antibody has been negatively preadsorbed using a non-phosphopeptide corresponding to the site of phosphorylation to remove antibody that is reactive with non-phosphorylated pRb. The final product is generated by affinity chromatography using a pRb-derived peptide that is phosphorylated at threonine 821. Figure 9 defines the specificity of the anti-Rb [pT^{821}]. SDS-PAGE on a 7.5% Tris-glycine gel resolved cell extracts, prepared from MCF-7 cells. The proteins were then transferred to PVDF. Membranes were incubated with 0.5 µg/mL anti-RB [pT^{821}], following prior incubation in the absence (a) or presence of the peptide immunogen (b), the non-phosphopeptide corresponding to the RB phosphopeptide (c), the phosphopeptides corresponding to threonine 356 (d), serine 807/811 (e), serine 249/threonine 252 (f), and serine 751 (g) on phospho-RB. After washing, membranes were incubated with goat $F(ab')_2$ anti-rabbit IgG alkaline phosphatase and bands were detected using the Tropix WesternStar™ detection method. The data show that only the phosphopeptide corresponding to this site blocks the antibody signal, therefore demonstrating the specificity of the anti-Rb [pT^{821}] antibody for this phosphorylated residue.

Total Rb [pan] Detection Antibody: the detection antibody is a monoclonal, clone G3-245, available commercially from BD/Pharmingen (San Diego, CA). It recognizes an epitope between amino acids 332-344 of Rb protein. This antibody will bind to both non-phosphorylated and phosphorylated forms of Rb protein.

Rb monoclonal antibody: the capture antibody [linked to the solid phase] is a monoclonal, clone 3C8, available commercially from QED Biosciences (San Diego, CA). It reacts with epitope on near the C-terminal end of the Rb protein (aa886-aa905). This antibody will bind to both non-phosphorylated and phosphorylated forms of Rb protein.

Total Rb and Rb [pT^{821}] ELISA: A concentration of 1.25 μg/mL of Rb monoclonal antibody in carbonate buffer, pH 9.4, was incubated at 100 μL/well in microtiter plates at 4°C overnight. The wells were washed with a PBS/Tween-20 solution three times followed by blocking on other sites on the plastic surface with a buffered solution containing unrelated proteins such as BSA for 2 hours at room temperature. Jurkat cell lysate containing phosphorylated Rb or non-phosphorylated recombinant Rb were added to the wells at various concentrations and incubated for 2 hour at room temperature. After three washings with Washing Buffer, the wells were incubated, respectively, with Rb [pT^{821}] PSSA and biotinylated Pan-Rb antibodies at the optimized concentrations (ranging from 0.1 to 1 μg/mL) for 1 hour at room temperature. The plates then were washed three times with Washing Buffer, followed by the addition of an HRP conjugated anti-rabbit IgG secondary antibody at 1:5000 dilution or 0.25 μg/mL of streptavidin-HRP for 1 hour at room temperature. After washing, 100 μL of Stabilized Chromogen was added to each well and then incubated for 20 minutes at room temperature in the dark. The OD values at 450 nm were measured following the addition of stop solution to each well.

Figure 10 shows studies to determine the specificity of the Rb [pT^{821}] ELISA. In the first study, solutions containing Rb protein at a concentration of 20 ng/mL from Jurkat, U2OS, and Colo205 were analyzed with the Rb [pT^{821}] ELISA kit, along with a solution containing 20 ng/mL purified full length Rb protein expressed in *E. coli* (non-phosphorylated). Figure 11 shows that the Rb protein isolated from the cell lines was strongly recognized. These data provide evidence that appropriate phosphorylation of the Rb protein is requisite for reactivity in this assay.

In the second study, specificity for threonine 821 was determined by peptide competition. The data presented in Figure 11 show that only the peptide corresponding to the region surrounding threonine 821, containing the phospho-threonine, could block the ELISA signal.

Kinase reactions for Rb: Natural sources for Rb were obtained for these studies from exponentially growing cells. Endogenous cellular kinases provided the phosphorylation of the natural Rb protein. Figure 12 shows the application of this ELISA to study kinase

reactions. Jurkat cells were grown in the presence of the kinase inhibitor, staurosporine, at various concentrations for 36 hours prior to lysis. Lysates were normalized for total Rb content using the BioSource Total Rb ELISA (catalog #KHO0011). Levels of Rb phosphorylation at threonine 821 were determined. These data show that staurosporine inhibits the phosphorylation of Rb at threonine 821, possibly through the inhibition of cdks.

EGFR System: This model system presents a cell surface receptor protein. This protein is large with multiple phosphorylation sites consisting of phospho-threonine, phospho-serine and phospho-tyrosine residues.

EGFR protein: Human EGFR protein was purified from human carcinoma A431 cells by affinity purification. The product is purchased from Sigma (St. Louis, MO cat # E-2645).

EGFR [pY^{1173}] PSSA: Rabbit antiserum was produced against a chemically synthesized phosphopeptide derived from the region of EGFR that contains tyrosine 1173. The sequence is conserved in human, mouse, and rat. Antibody was purified from serum by sequential epitope-specific chromatography. The antibody has been negatively preadsorbed using (i) a non-phosphopeptide corresponding to the site of phosphorylation to remove antibody that is reactive with non-phosphorylated EGFR enzyme, and (ii) a generic tyrosine phosphorylated peptide to remove antibody that is reactive with phospho-tyrosine (irrespective of the sequence). The final product is generated by affinity chromatography using an EGFR-derived peptide that is phosphorylated at tyrosine 1173. Figure 13 illustrates the specificity of the EGFR PSSA [pY^{1173}]. Cell extracts prepared from NIH3T3 cells expressing EGFR were starved for 30 hours, then stimulated for 10 minutes with 30 ng/mL EGF (+), or left unstimulated (-), then resolved by SDS-PAGE on a 6% Tris-glycine gel, and transferred to nitrocellulose. Membranes were incubated with 0.50 μg/mL anti-EGFR [pY^{1173}] antibody, following prior incubation in the absence (lanes 1& 2), or presence of the peptide immunogen (lanes 3 & 4), or the non-phosphopeptide corresponding to the EGFR phosphopeptide (lanes 5 & 6). After washing, membranes were incubated with goat $F(ab')_2$ anti-rabbit IgG alkaline phosphatase and bands were detected using the Tropix WesternStar™ detection method. The data show that only the phosphopeptide

corresponding to this site blocks the antibody signal, demonstrating the specificity of the anti-EGFR [pY^{1173}] antibody for this phosphorylated residue.

EGFR [pY^{845}] PSSA: Prepared essentially as EGFR [pY^{1173}] PSSA but using chemically synthesized phosphopeptides from the region that contains tyrosine 845.

EGFR [Pan] monoclonal antibody: The capture antibody is a mouse monoclonal antibody, clone 199.12, available commercially from Neomarkers, Inc. (Union City, CA). It is specific for human EGFR and does not react with HER2/neu, HER3 and HER4. This antibody will bind to both non-phosphorylated and phosphorylated forms of EGFR protein.

EGFR [Pan] Detection Antibody: This rabbit antibody was prepared by immunization with a synthetic peptide corresponding to C-terminus of human EGFR. The antibody was purified using protein A affinity column. It shows no cross-reactivity with HER2/neu, HER3 and HER4.

EGFR PSSA and Full Length ELISA: A concentration of 2.5 μg/mL of pan-EGFR monoclonal antibody in carbonate buffer, pH 9.4, was incubated at 100 μL/well in microtiter plates at 4°C overnight. The wells were washed with a PBS/Tween-20 solution three times followed by blocking on other sites on the plastic surface with a buffered solution containing unrelated proteins such as BSA for 2 hours at room temperature. Autophosphorylated EGFR or non-phosphorylated EGFR were added to the wells at various concentrations and incubated for 1 hour at room temperature. After three washings with Washing Buffer, the wells were incubated, respectively, with EGFR [pY^{845}] PSSA, EGFR [pY^{1173}] PSSA, and Pan-EGFR antibodies at the optimized concentrations (ranging from 0.1 to 1 μg/mL) for 1 hour at room temperature. The plates then were washed three times with Washing Buffer, followed by the addition of an HRP conjugated anti-rabbit IgG secondary antibody at 1:2000 dilution for 1 hour at room temperature. After washing, 100 μL of Stabilized Chromogen was added to each well and then incubated for 20 minutes at room temperature in the dark. The OD values at 450 nm were measured following the addition of stop solution to each well.

The specificity of the EGFR [pY^{1173}] ELISA for tyrosine residue 1173 was determined by peptide competition. The data presented in Figure 14 show that only the peptide corresponding to the region surrounding tyrosine residue 1173, containing the phospho-threonine, could block the ELISA

Kinase Reactions: (autophosphorylation)

EGFR was incubated (auto-phosphorylated) in the buffer of 15 mM HEPES (pH7.4), 6 mM MnCl2 and 15 mM MgCl2 containing 1uM ATP for 30 minutes at 30oC.

Figure 15 demonstrates the response curve of phosphorylation of EGFR in A431 cells after treatment with EGF at 1-500 ng/mL for 10 minutes. The phosphorylation of EGFR tyrosine was detected with EGFR [pY^{1173}] ELISA.

Figure 16 demonstrates use of the described invention to detect protein kinase activity associated with EGFR at residue pY845 and inhibition of that activity by a protein kinase inhibitor. 2 ng/ vial of purified human EGFR was incubated (auto-phosphorylated) in the buffer of 15 mM HEPES (pH7.4), 6 mM MnCl2 and 15 mM MgCl2 containing 1uM ATP for 30 minutes at 30oC. For phosphorylation inhibition of EGFR [pY^{845}], tyrosine kinase inhibitor PD158780 (Calbiochem, cat #. 513035) was added to the vials at indicated concentration. EGFR [pY^{845}] phosphorylation was measured at 4ng/mL using EGFR [pY^{845}] phosphorylation site specific ELISA.

Table 1: List signal transduction proteins the phosphorylation of which can be determined by methods of the present invention.

TABLE 1

Examples of Signal Transduction Proteins

Protein

A

alpha-actinin
alpha-synuclein
ABL/c-Abl (Abelson nonreceptor protein tyrosine kinase)
Acetylcholine Receptor
Ack nonreceptor protein tyrosine kinase;
Akt/PKB serine/threonine protein kinase
AP-1 (Activator protein-1 jun/fos dimeric transcription factors
AP-2 (Activator protein-2 transcription factor
Apaf-1 (Apoptosis protease-activating factor-1)
Apaf-2 (Apoptosis protease-activating factor-2/cytochrome C)
Apaf-3 (Apoptosis protease-activating factor-3/caspase-9
Arp2/3 (Actin related protein)
Atf-1 (Activating transcription factor-1)
Atf-2 (Activating transcription factor-2)
Atf-3 (Activating transcription factor-3)
Atf-4 (Activating transcription factor-4)
ATM (Ataxia Telangiectasia Mutated. Protein)

B

B-ATF nuclear basic leucine zipper protein/transcription factors
Bad
Bak
Bax
Bcl-2 (B-cell chronic lymphocytic leukemia 2)
Bcl-xL
Bcl-xS
BCR/ABL protein tyrosine kinase
beta-Catenin
BID (BH-3 Interacting Death Domain)
Blk (B Lymphocyte Src non-receptor protein tyrosine kinase family member)
BMK-1 (Big Map Kinase/ERK5)
Btk (Bruton's Tyrosine Kinase)

C

Cadherin
CADTK (calcium activated protein tyrosine kinase/Cakbeta/Pyk2/FAK2/RAFTK)
CAK (Cdk-Activating Kinase)
Cak-beta (Cell adhesion kinase beta/ CADTK/Pyk2/FAK2/RAFTK)
caldesmon
calmodulin
calpain cysteine proteases
CaM kinase II (Calmodulin-dependent protein kinase II)
CB1 (Cannabinoid Receptor 1)

CB2 (Cannabinoid Receptor 2)
caspase-2 (Cysteine Aspartyl Protease-2/ ICH-1/NEDD-2)
caspase-3 (Cysteine Aspartyl Protease-3/LICE/CPP32/YAMA/apopain/SCA-1)
caspase-8 (Cysteine Aspartyl Protease-8/MACH/FLICE/Mch5)
caspase-9 (Cysteine Aspartyl Protease-9/ICE-LAP6/Mch6/APAF-3)
Caveolin 1, 2, and 3)
CD45 transmembrane tyrosine phosphatase
CD45AP (CD45-associated protein)
c-fos transcription factor
CDK1/cdc2 (Cyclin-dependent kinase-1)
CDK2 (Cyclin dependent kinase-2)
CDK4 (Cyclin dependent kinase-4)
CDK5 (Cyclin dependent kinase-5)
c-Jun transcription factor
c-myc transcription factor
Cortactin
COX-2 (Cyclooxygenase-2/prostaglandin-endoperoxide synthase-2)
c-kit receptor protein
c-raf protein serine/threonine kinase
CREB transcription factor
Crk SH2 and SH3 domain-containing adaptor protein
CSK (Carboxyl-terminal Src Kinase)
cytochrome-c

D

DAPK (Death Associated Protein Kinase)
desmin
DNA-PK (DNA dependent protein kinase)

E

E2F-1 DNA binding protein
EGF-R (Epidermal Growth Factor Receptor)
eIF-2alpha (Eukaryotic translation Initiation Factor 2alpha)
ERK1/MAPK (Extracellular signal-Regulated/Mitogen-Activated Protein Kinase 1)
ERK2/MAPK (Extracellular signal-Regulated/Mitogen-Activated Protein Kinase 2)
ERK3 (Extracellular signal-Regulated/p62 Mitogen-Activated Protein Kinase 3)
ERK4 (Extracellular signal-Regulated Protein Kinase 4)
ERK5 (Extracellular signal-Regulated Protein Kinase 5/Big MAP Kinase 1)
ERK6 (Extracellular signal-Regulated Protein Kinase 6/p38gamma)
ERK7 (Extracellular signal-Regulated Protein Kinase 7)
ERK5 (Extracellular signal-Regulated Protein Kinase 8)

F

F-actin
FADD (Fas-associated Death Domain)
FAK (Focal Adhesion Kinase/pp125FAK)
FAS (FAS-Ligand Receptor)
Fgr non-receptor Src family tyrosine kinase
Fos B
Fra-1 (Fos-related antigen-1)
Fra-2 (Fos-related Antigen-2)
FRK (Fos-Regulating Kinase)
FYB (Fyn binding protein)
Fyn non-receptor Src family tyrosine kinase

G

Gab 1 (Grb2-associated binder 1)
Gab 2 (Grb2-associated binder 2)
GCK (Germinal Center Kinase)
GEF (Guanine nucleotide Exchange Factor)
Giα inhibitory guanine nucleotide regulatory protein
Giβ inhibitory guanine nucleotide regulatory protein
Giγ inhibitory guanine nucleotide regulatory protein
Gq/11 guanine nucleotide-binding protein
Gq/11β guanine nucleotide-binding protein
Gq/11γ guanine nucleotide-binding protein
Grb2 (Growth factor Receptor Binding protein-2)
Grk2 (G protein-coupled Receptor Kinase)
GSK-3α (Glycogen Synthase Kinase 3alpha)
GSK-3β (Glycogen Synthase Kinase 3beta)

H

Hck (Hematopoietic cell kinase)
HGF-R (Hepatocyte growth factor receptor)
Hrk (3-Hydroxy-3-methyl glutaryl-coenzyme A Reductase Kinase)

I

IkappaB alpha NFkB inhibitory protein
IkappaB beta NFkB inhibitory protein
IKKalpha (IkB kinase alpha)
IKKbeta (IkB kinase beta)
IKKgamma (IkB kinase gamma/NEMO)
IGF-1 receptor (Insulin-like growth factor-I receptor)
Insulin receptor
Integrins
Integrin-Associated Protein (IAP/CD47)
IRAK (Interleukin-1 Receptor-Associated Kinase)
IRK (Insulin Receptor Kinase)
IRS-1 (Insulin Receptor Substrate 1)
IRS-2 (Insulin Receptor Substrate 2)

J

JAB1 (Jun-Activation domain Binding protein 1)
JAK1 (Janus Activating Kinase 1)
JAK2 (Janus Activating Kinase 2)
JAK3 (Janus Activating Kinase 3)
JNK1/SAPKγ (c-Jun amino-terminal kinase 1/Stress-Activated Protein Kinase γ)
JNK2/SAPKβ (c-Jun amino-terminal kinase 2/Stress-Activated Protein Kinase β)
JNK3/SAPKα (c-Jun amino-terminal kinase 3/Stress-Activated Protein Kinase α)

L

LAT (Linker for Activation of T cells)
Lck non-receptor Src family protein tyrosine kinase
Lyn non-receptor Src family protein tyrosine kinase

M

MEF2c transcription factor
MEK1 (Mitogen-activated ERK-activating Kinase 1)
MEK2 (Mitogen-activated ERK-activating Kinase 2)
MEK3 (Mitogen-activated ERK-activating Kinase 3)
MEK4 (Mitogen-activated ERK-activating Kinase 4)

MEK5 (Mitogen-activated ERK-activating Kinase 5)
MEKK1 (MEK kinase 1)
Met (c-met/HGF-receptor)
MKP 1 (MAP Kinase Phosphatase 1)
MKP 2 (MAP Kinase Phosphatase 2)
MKP 3 (MAP Kinase Phosphatase 3)
MKP 4 (MAP Kinase Phosphatase 4)
MKP 5 (MAP Kinase Phosphatase 5)
MKP 6 (MAP Kinase Phosphatase 6)
MLCK (Myosin light chain kinase)
MuSK (Muscle specific serine/threonine kinase)
Myosin
MLCK PPase (Myosin Light Chain Kinase Phosphatase)

N
Beta-NAP (Beta-Neuron Adaptor Protein/AP-3)
NAT1/DAP-5 (Novel APOBEC-1 Target no.1/Death-Associated Protein-5)
NCK SH2 and SH3 domains-containing transforming protein
Nek2 (Nima-related Kinase2)
NFAT-1 (Nuclear Factor of Activated T-cells)
NfkappaB (Nuclear Factor Kappa B transcription factor)
NIK (NFkappaB Inducing Kinase)
NTK (Nervous Tissue and T cell Kinase)

P
p130cas
p190RhoGAP GTPase
P2Y2 purinoceptor
p36 CAK assembly/activation factor
p38 (ERK6 MAPK/SAPK)
p38d (SAPK4)
p53 Tumor suppressor gene.
p58 IPK (Inhibitor of the interferon-induced double-stranded RNA-activated Protein Kinase, PKR)
p62dok GAP-associated protein
p62 lck ligand/ZIP
p68 kinase
p96
PAK1 (p21-Activated protein Kinase 1)
PAK2 (p21-Activated protein Kinase 2)
PAK3 (p21-Activated protein Kinase 3)
PARP (Poly(ADP-Ribose) Polymerase)
Paxillin
PCNA (Proliferating Cell Nuclear Antigen)
PDGF Receptor (Platelet Derived Growth Factor Receptor)
PDK1 (Phosphoinositide-Dependent Kinase-1)
PDK-2 (Phosphoinositide-Dependent Kinase-2/ Integrin-linked kinase)
PECAM-1 (Platelet-Endothelial Cell Adhesion Molecule-1)
PI3K (Phosphatidyl Inositol-3-Kinase)
PIAS (Protein Inhibitors of Activated STATs)
PITP alpha (Phosphatidylinositol Transfer Protein alpha)
PKA alpha/cAMP-dependent protein kinase
PKB (Protein kinase B)
PKC alpha (Protein Kinase C alpha)
PKC beta (Protein Kinase C beta)
PKC delta (Protein Kinase C delta)
PKC gamma (Protein Kinase C gamma)
PKD (Protein Kinase D)
PKR (Protein Kinase R or double-stranded RNA-activated protein kinase)

PLC-gamma1 (Phospholipase C-gamma1)
PRK (Proliferation Related Kinase)
PTEN (MMAC1 tumor suppressor gene/protein phosphatase)
Pyk2 (CAKbeta/FAK2/RAFTK) Protein tyrosine Kinase

R

Rac/cdc42 GTPase
Raf1 (C-raf) serine/threonine protein kinase
A-Raf serine/threonine protein kinase
B-raf serine/threonine kinase
V-Raf viral serine/threonine protein kinase
RAFTK (Related Adhesion Focal Tyrosine Kinase)
RAIDD (RIP-Associated ICH-1/CED-3 homologous protein with a Death Domain)
Rap2 GTPase
Rap1-GAP (C3G) inactivator of Rap-1
Rapsyn
Ras GTPase
Rb (Retinoblastoma tumor suppressor protein)
Rho Small molecular weight GTPase
RIP (Receptor Interacting Protein)
ROCK (Rho-activated kinase)

S

S6k (S6 Kinase)
Shc
SHIP (SH2 domain containing inositol phosphatase)
SH-PTP1 Protein Tyrosine Phosphatase
SH-PTP2 Protein Tyrosine Phosphatase
SIRPalpha1 (Signal Related Protein Alpha)
SIP1 (Smad Interacting Protein 1)
Smad2 (Sma and Mad-related 2)
Smad3 (Sma and Mad-related 3)
Smad5 (Sma and Mad-related 5)
Smad7 (Sma and Mad-related 7)
SOCS-1 (Suppressor of Cytokine Signaling-1)
SOCS-2 (Suppressor of Cytokine Signaling-2)
SOCS-3 (Suppressor of Cytokine Signaling-3)
SOS (Son of Sevenless)
Src non-receptor tyrosine kinase
SRF (Serum Response Factor)
SRPK1 (SR protein-specific Kinase1)
SRPK2 (SR protein-specific Kinase2)
STAT1alpha (Signal Transducer and Activator of Transcription 1)
STAT2 (Signal Transducer and Activator of Transcription 2)
STAT3 (Signal Transducer and Activator of Transcription 3)
STAT4 (Signal Transducer and Activator of Transcription 4)
STAT5alpha (Signal Transducer and Activator of Transcription 5alpha)
STAT5beta (Signal Transducer and Activator of Transcription 5 beta)
STAT6 (Signal Transducer and Activator of Transcription 6)
Syk (Spleen tyrosine kinase)
Syndecans transmembrane proteoglycan

T

Tak1 (TGF-b1 activated kinase)
Talin
TANK/I-TRAF (TNF Receptor Activating Factor)
Tau microtubule-associated protein
TBK-1/T2K (TANK Binding Kinase 1)

Tensin
TNF-RI (Tumor Necrosis Factor Receptor I)
TRADD (TNF-Receptor Associated Death Domain protein)
TRAF1 (TNF-Receptor Associated Factor 1)
TRAF2 (TNF-Receptor Associated Factor 2)
TRAF3 (TNF-Receptor Associated Factor 3)
TRAF4 (TNF-Receptor Associated Factor 4)
TRAF5 (TNF-Receptor Associated Factor 5)
TRAF6 (TNF-Receptor Associated Factor 6)

TrkA protein tyrosine receptor kinase A
TrkB protein tyrosine receptor kinase B
TrkC protein tyrosine receptor kinase C

V
VEGF-receptor (vascular endothelial growth factor receptor, types 1, 2, 3)
Vinculin

W
WASP (Wiskott-Aldrich Syndrome Protein)

Z
ZIP (Zeta Interacting Protein)
ZIP kinase (zipper serine/threonine kinase)
ZRP-1 (Zyxin Related Protein
Zyxin

Antibodies of the present invention are also useful for inactivating phosphorylated polypeptides for therapeutic purposes. The examples illustrate the present invention and are not intended to limit the invention in spirit or scope. Similarly, the description of these reagents and methods can be used in an inverse function to analyze the activity of protein specific phosphatases, enzymes that remove phosphate groups from specific amino acid residues.

What is claimed:

1. A method for measuring phosphokinase activity on a protein comprising:

(a) subjecting a protein to a phosphokinase to phosphorylate a phosphorylation site on the protein

(b) providing an antibody specific to a phosphorylated phosphorylation site on the protein;

(c) contacting the protein from (a) with the antibody of (b); and

(d) detecting the antibody bound to the phosphorylation site.

2. A method according to claim 1 wherein the protein is the Tau protein.

3. A method according to claim 2 wherein the phosphokinase is GSK-3β, PKA, PKC, CDK-5, MARK, JNK, p38MAPK, or casein kinase II.

4. The method according to claim 2 wherein the antibody is specific to a phosphorylated site at a specific location in the Tau protein , selected from the sites shown in Table II.

5. A method according to claim 1 wherein the protein is selected from the proteins in Table 1.

6. The method of claim 1 wherein the protein is EGFR.

7. The method of claim 1 wherein the protein is Rb.

8. An antibody prepared from a polypeptide immunogen having a phosphorylated serine.

9. An antibody raised to a polypeptide immunogen having a phosphorylated threonine.

10. An antibody raised to a polypeptide immunogen having a phosphorylated tyrosine.

11. An antibody specific for sequence ID #1.

12. An antibody specific for sequence ID #2.

13. An antibody to EGFR phosphorylated tyrosine 1173 or tyrosine 845 site.

14. An antibody to phosphorylated threonine 821 site.

15. A kit for the measurement of phosphokinase activity on a protein comprising

(a) a first pan antibody specific and that binds to both phosphorylated and non-phosphorylated forms of the protein ;
(b) a second pan antibody that binds to an independent site on the protein from the first pan antibody, wherein the second pan antibody is labeled;
(c) non-phosphorylated and phosphorylated protein standards;
(d) a phosphorylation site-specific antibody (PSSA) which binds to the protein only when the target site on the protein is phosphorylated and wherein the PSSA antibody is labeled; and
(e) buffers.

16. A kit according to claim 11 where the protein is Tau.

17. A kit according to claim 11 where the protein is selected from the proteins listed in Table I.

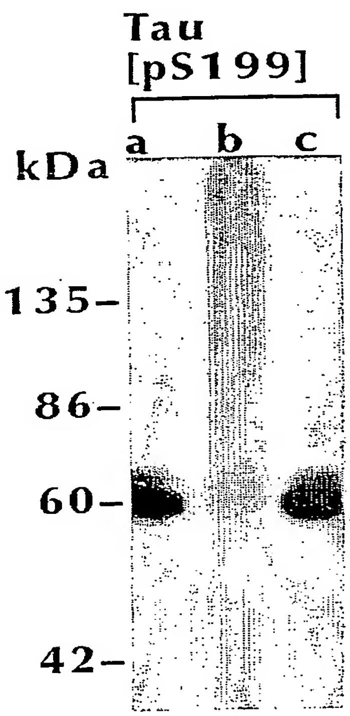
18. A kit for the measurement of different kinase activities on a protein by quantitating phosphorylation site profiles comprising:

(a) a first pan antibody that binds to both phosphorylated and non-phosphorylated forms of the protein;
(b) a second pan antibody that binds to an independent site on the protein from the first pan antibody and wherein the second pan antibody is labeled;
(c) protein standards for the non-phosphorylated and phosphorylated forms of the protein;
(d) two or more PSSAs which bind to the protein only when the target sites on the protein which are phosphorylated wherein antibody is labeled so as to be ; and
(e) buffers.

19. A kit according to claim 14 where the protein is Tau.

20. A kit according to claim 14 where the proteins are as defined in Table I.

Figure 1



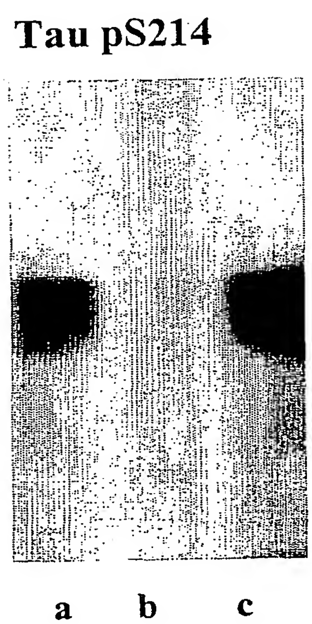


FIGURE 2

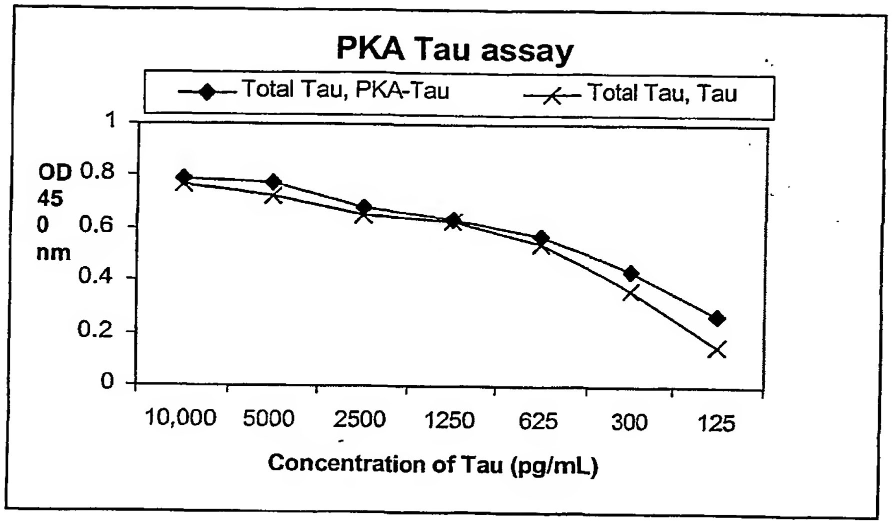


FIGURE 3a

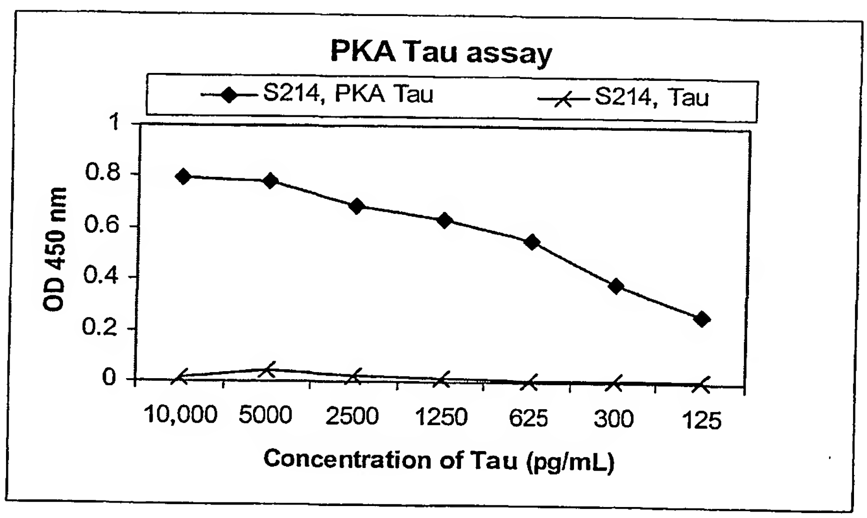


FIGURE 3b

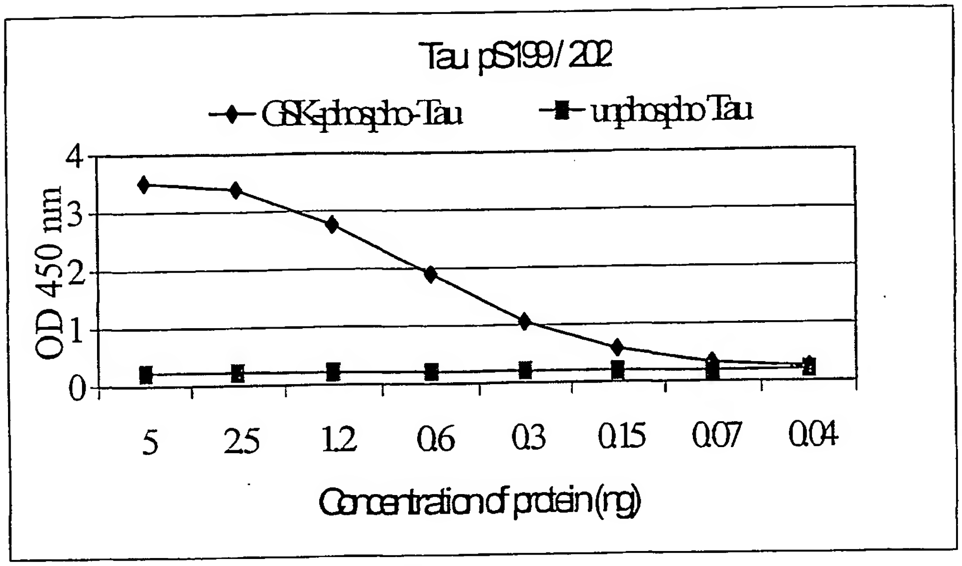


Figure 4a

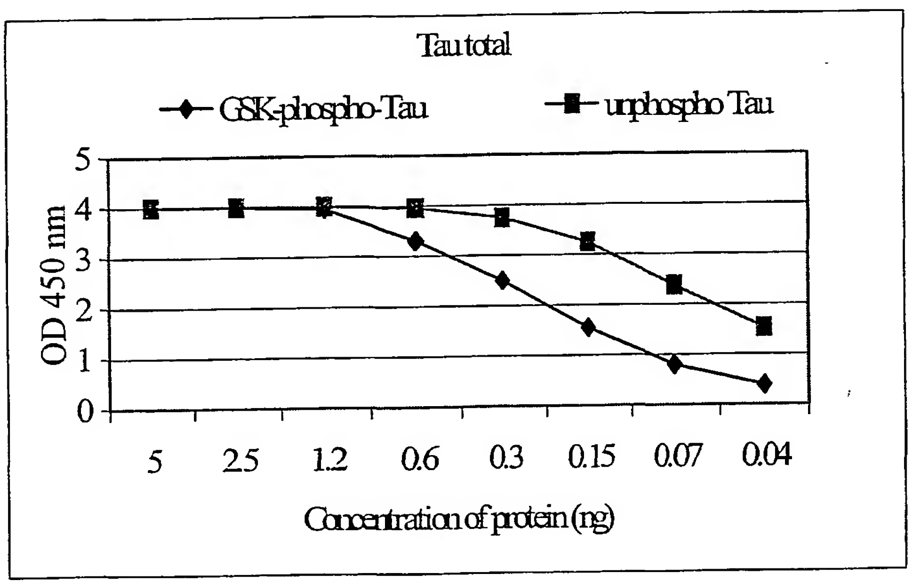


Figure 4b

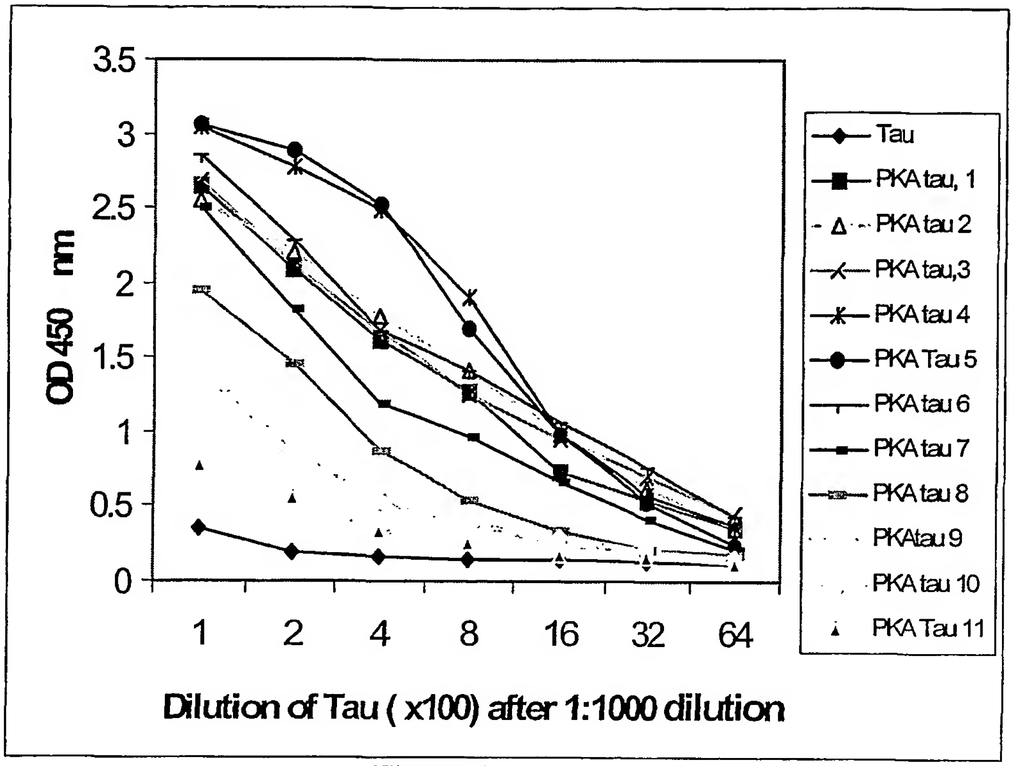

3.5
3
2.5
2
1.5
1
0.5
0
OD 450 nm
1
2
4
8
16
32
64
Dilution of Tau (x100) after 1:1000 dilution
Tau
PKA tau, 1
PKA tau 2
PKA tau,3
PKA tau 4
PKA Tau 5
PKA tau 6
PKA tau 7
PKA tau 8
PKAtau 9
PKA tau 10
PKA Tau 11

Figure 5

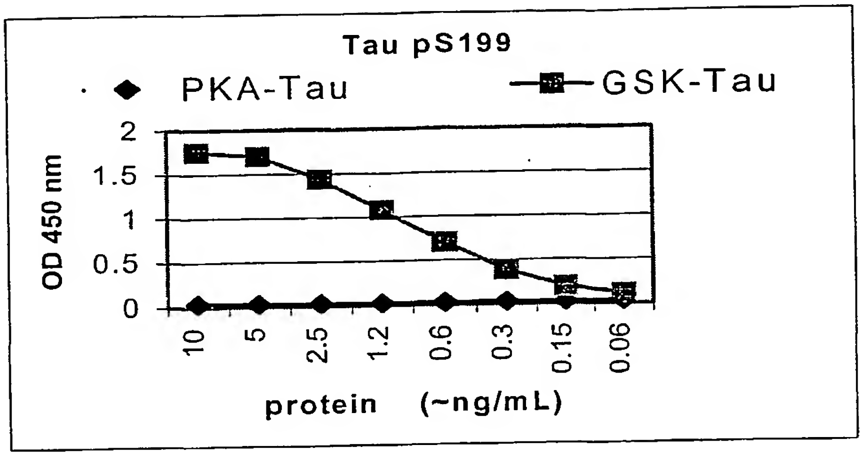


Figure 6a

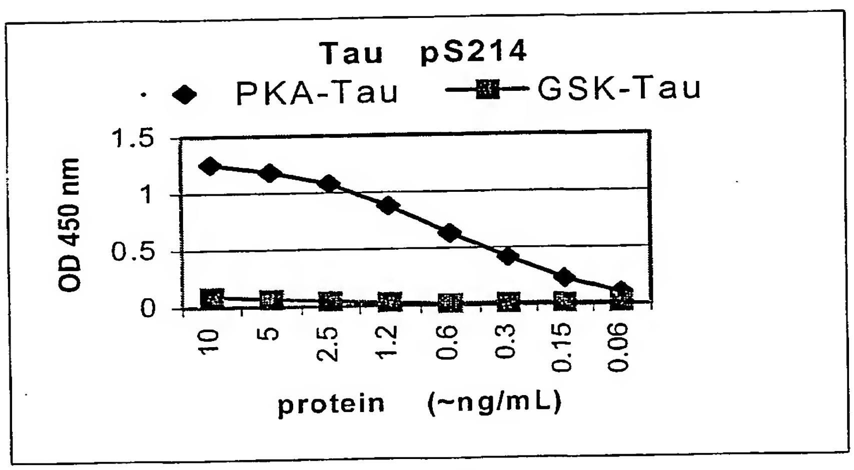


Figure 6b

Figure 7

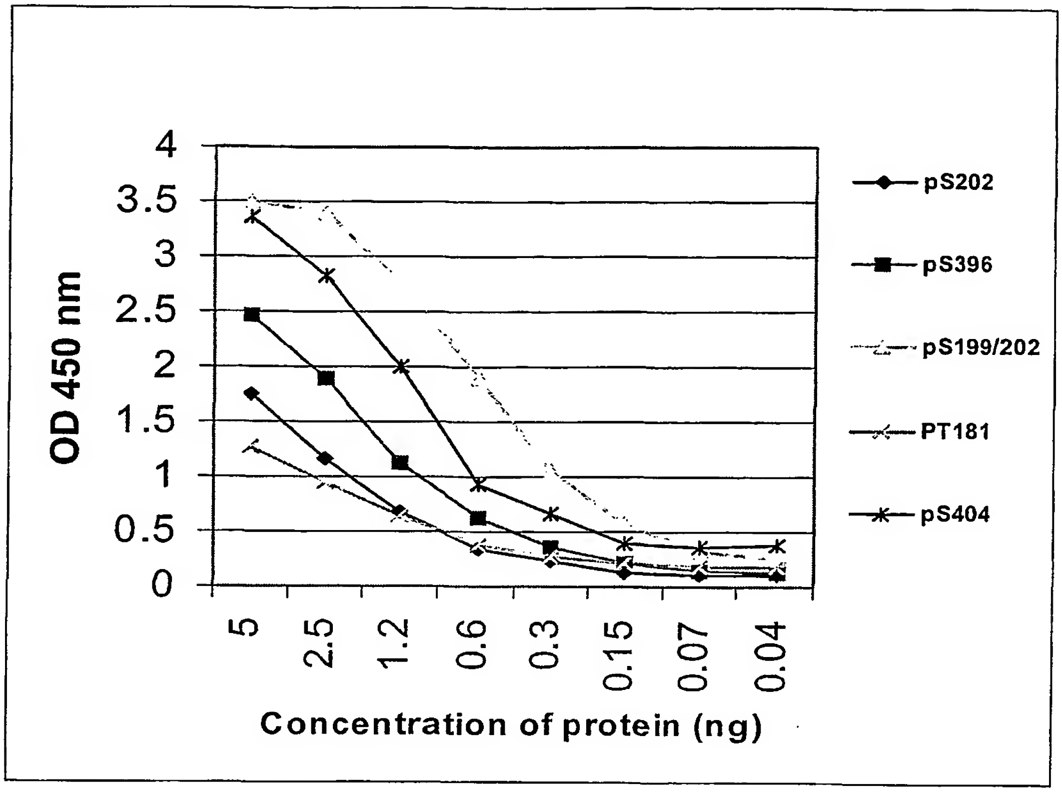

OD 450 nm
4
3.5
3
2.5
2
1.5
1
0.5
0
5
2.5
1.2
0.6
0.3
0.15
0.07
0.04
Concentration of protein (ng)
pS202
pS396
pS199/202
PT181
pS404

Figure 8

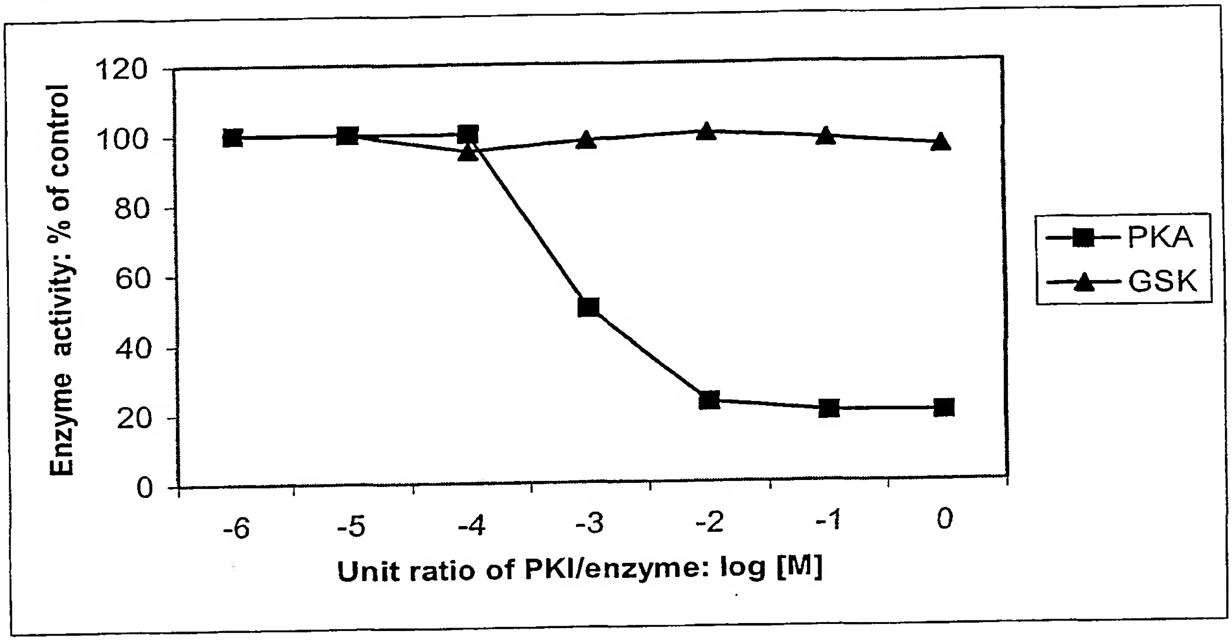


Figure 9

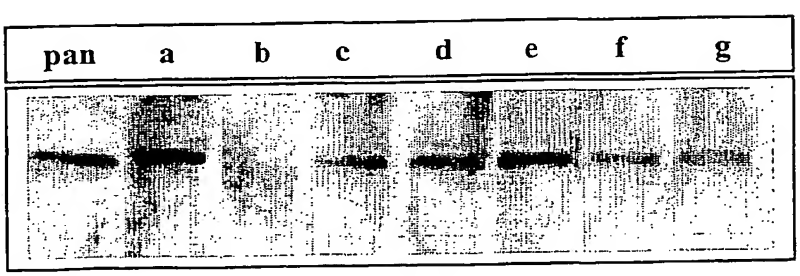


Figure 10

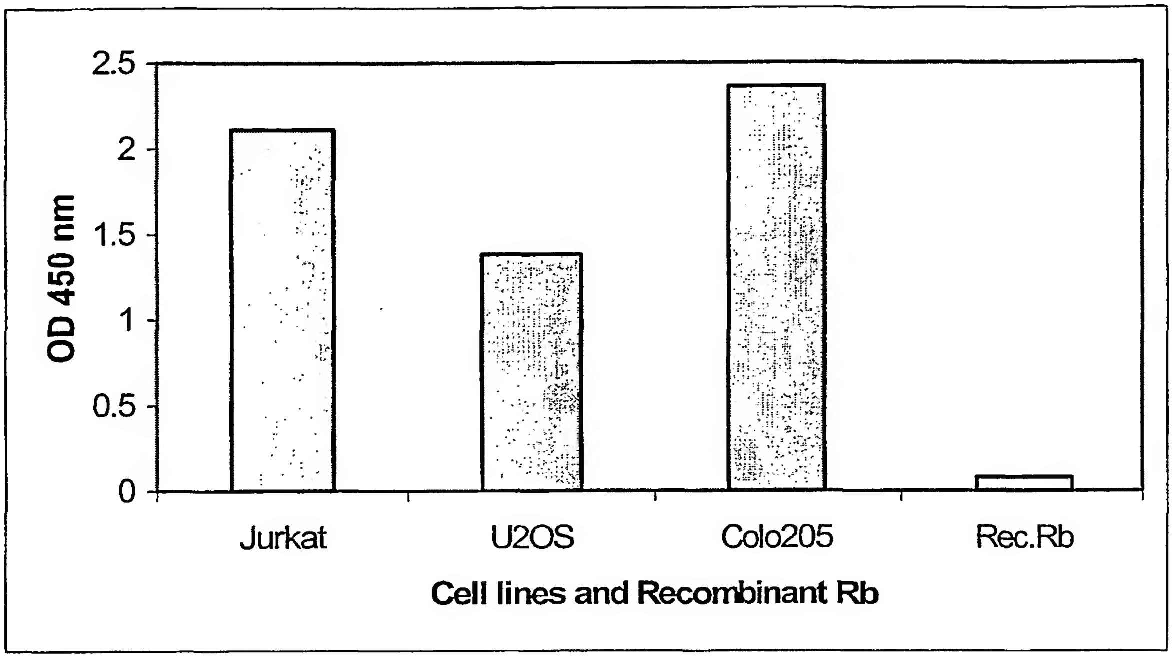

2.5
2
1.5
1
0.5
0
OD 450 nm
Jurkat
U2OS
Colo205
Rec.Rb
Cell lines and Recombinant Rb

Figure 11

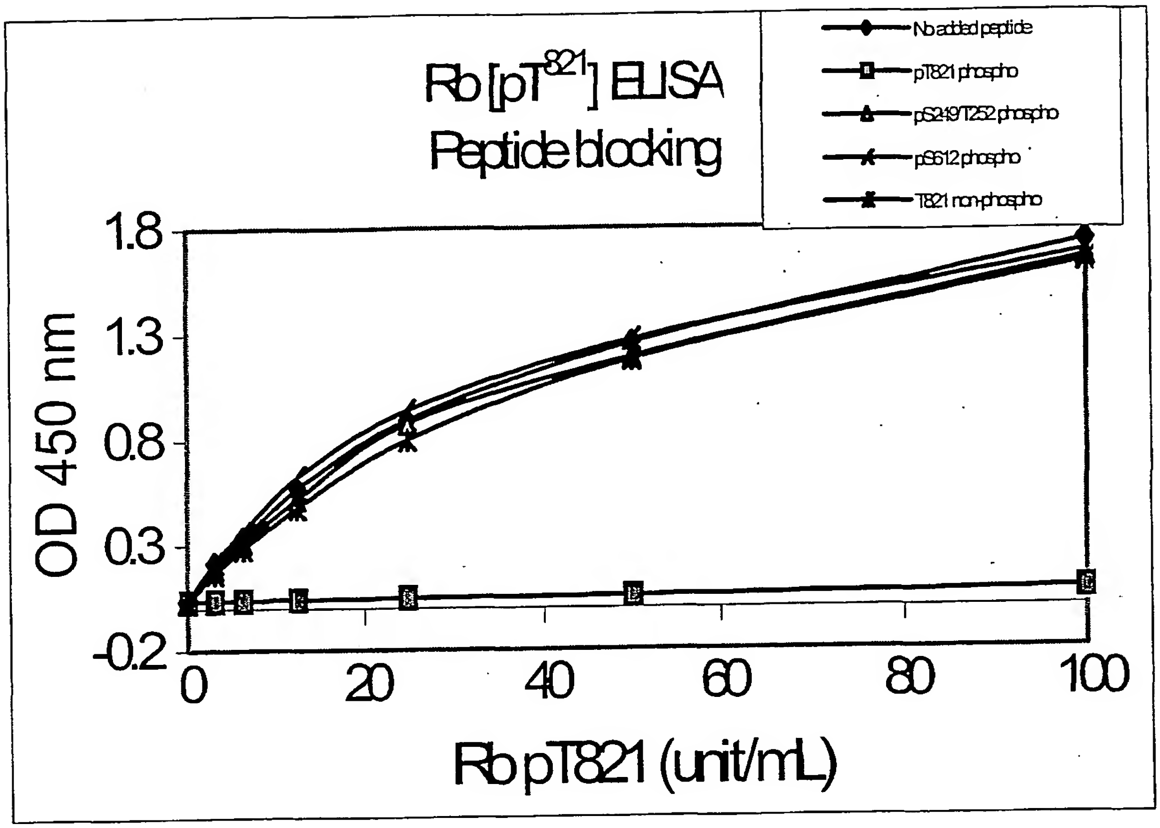

Rb [pT821] ELISA
Peptide blocking
No added peptide
pT821 phospho
pS249/T252 phospho
pS612 phospho
T821 non-phospho
OD 450 nm
1.8
1.3
0.8
0.3
-0.2
0
20
40
60
80
100
Rb pT821 (unit/mL)

Figure 12

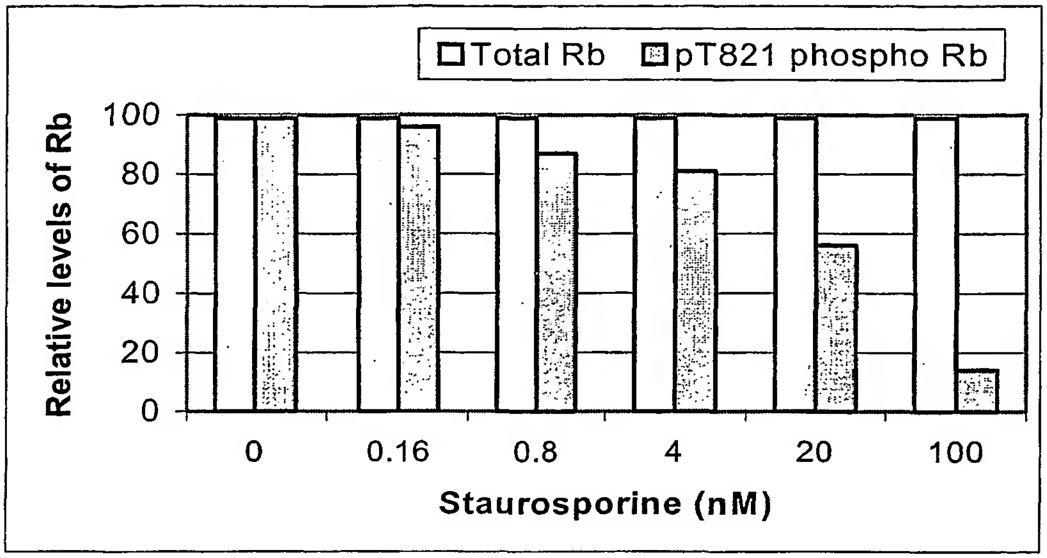
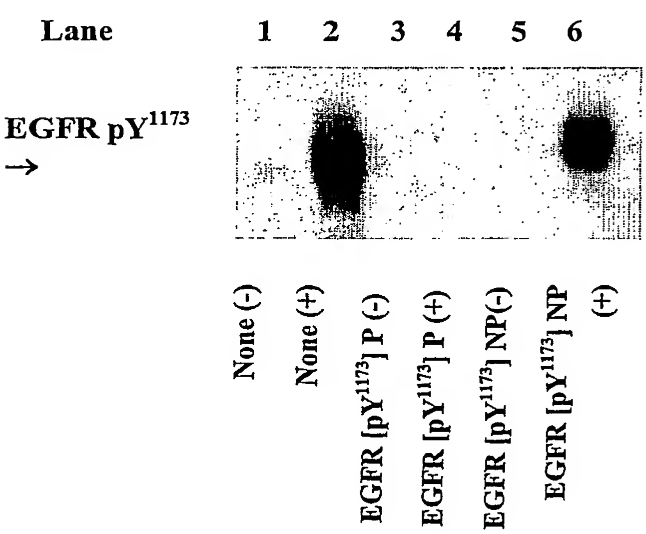


Figure 13



Peptide Used in Competition

Figure 14

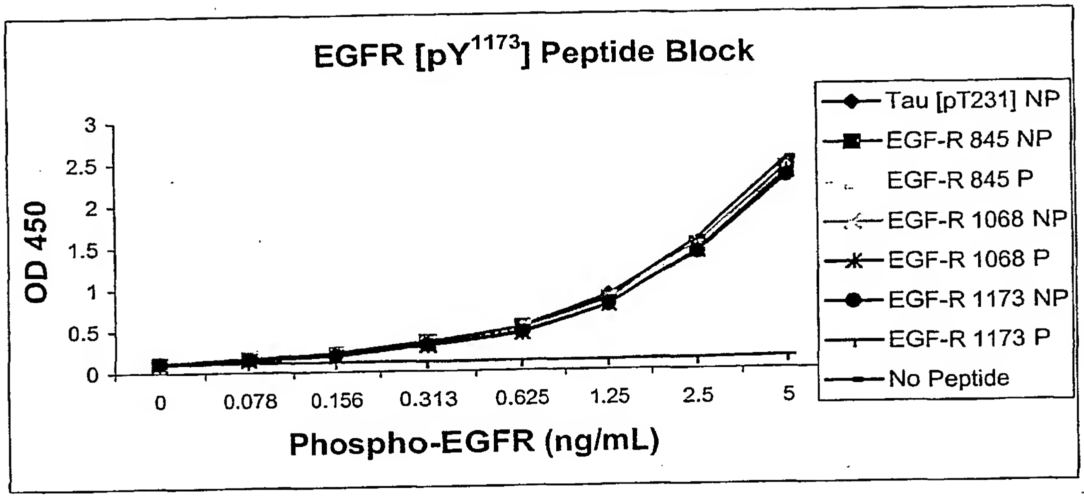

EGFR [pY1173] Peptide Block
OD 450
3
2.5
2
1.5
1
0.5
0
0
0.078
0.156
0.313
0.625
1.25
2.5
5
Phospho-EGFR (ng/mL)
Tau [pT231] NP
EGF-R 845 NP
EGF-R 845 P
EGF-R 1068 NP
EGF-R 1068 P
EGF-R 1173 NP
EGF-R 1173 P
No Peptide

Figure 15

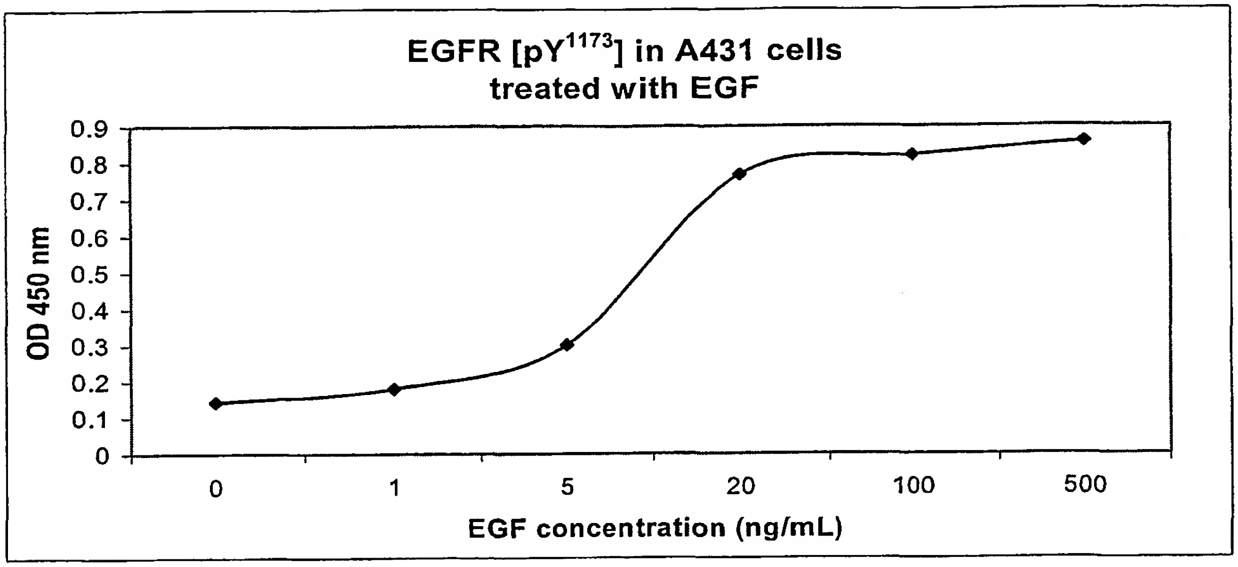
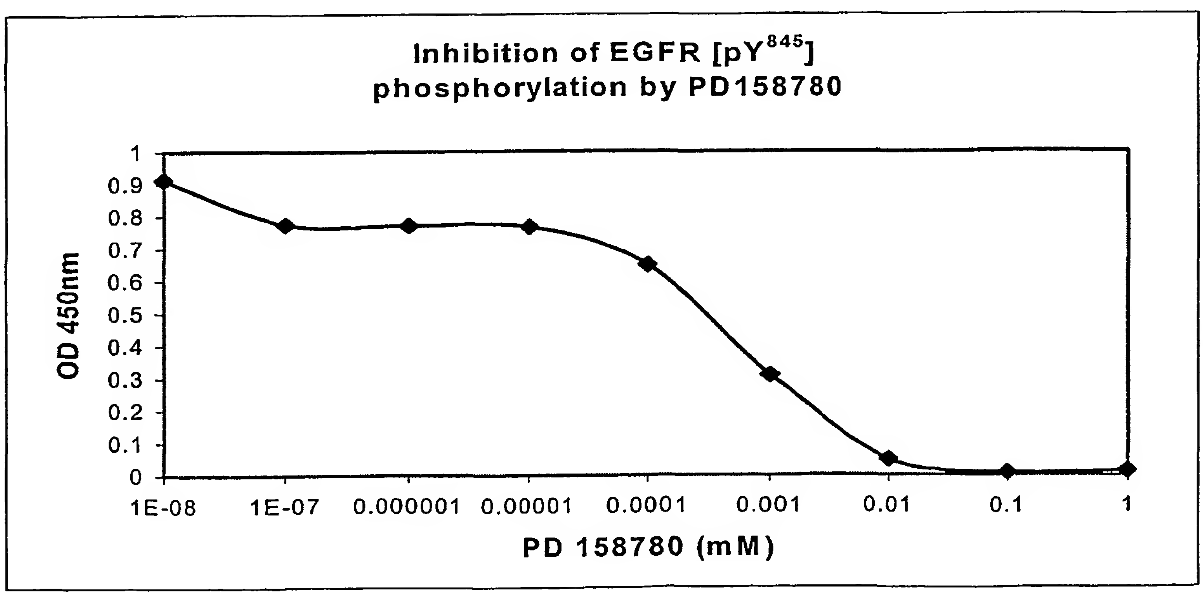


Figure 16



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 April 2002 (04.04.2002)

PCT

(10) International Publication Number
WO 02/027017 A3

(51) International Patent Classification⁷: G01N 33/68, C12Q 1/48, C07K 16/18, 14/47, 14/71

(21) International Application Number: PCT/US01/30186

(22) International Filing Date: 27 September 2001 (27.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/235,620 27 September 2000 (27.09.2000) US

(71) Applicant: BIOSOURCE INTERNATIONAL [US/US]; 820 Flynn Road, Camarillo, CA 93012 (US).

(72) Inventors: REAGAN, Kevin, J.; 5833 Middle Crest Dr., Agoura Hills, CA 91301 (US). SCHAEFFER, Erik; 12 Greystone Lane, Hopkinton, MA 01748 (US). WANG, Jimin; 657 Camino Del Mar, Thousand Oaks, CA 91320 (US).

(74) Agent: MCDONNELL, John, J.; McDonnell Boehnen Hulbert & Berghoff, 300 South Wacker Drive, Chicago, IL 60606 (US).

(81) Designated States *(national)*: CA, JP.

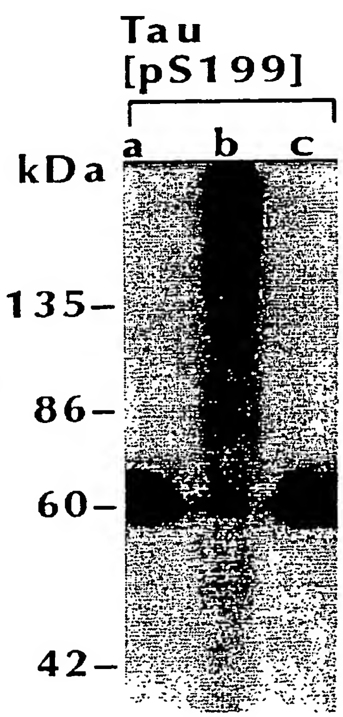
(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *with international search report*

(88) Date of publication of the international search report: 16 January 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD FOR QUANTIFYING PHOSPHOKINASE ACTIVITY ON PROTEINS



(57) **Abstract:** The invention involves a method for measuring phosphorylation of proteins and, as such, is an indicator of protein kinase activity. The method involves the *in vitro* phosphorylation of a target protein but subjecting that protein (non-phosphorylated) to reaction mixture containing all reagents, including phosphokinase which allow the creation of a phosphorylated form of protein. The phostphorylated protein is measured by contacting it with an antibody specific for the phosphorylation sites(s). The invention includes antibodies useful in practicing the methods of the invention. The invention particularly relates to phosphorylation of Tau, Rb and EGFR proteins and antibodies specific for the site of phosphorylation of the Tau, Rb or EGFR proteins.

WO 02/027017 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/30186

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 C12Q1/48 C07K16/18 C07K14/47 C07K14/71

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 24473 A (HOSCHUETZKY HEINZ ;WALTER ULRICH (DE); EIGENTHALER MARTIN (DE); HO) 20 May 1999 (1999-05-20) page 20, line 30 -page 21, line 21 ---	8
X	ZHENG-FISCHHOEFER QINGYI ET AL: "Sequential phosphorylation of Tau by glycogen synthase kinase-3beta and protein kinase A at Thr212 and Ser214 generates the Alzheimer-specific epitope of antibody AT100 and requires a paired-helical-filament-like conformation." EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 252, no. 3, March 1998 (1998-03), pages 542-552, XP002202096 ISSN: 0014-2956 *the whole document; see esp. Fig. 2* --- -/--	8,11,12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 September 2002	16. 10. 02

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Thiele, U

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 544 942 A (MAX PLANCK GESELLSCHAFT) 9 June 1993 (1993-06-09) claim 13; table 1	8
X	WO 96 04309 A (INNOGENETICS NV ;VANMECHELEN EUGEEN (BE); VOORDE ANDRE VAN DE (BE)) 15 February 1996 (1996-02-15) page 22 -page 23; claim 1	8
X	"Catalog No.: SA-307; Product: Anti-phospho-EGFR (pY1173)" PRODUCT DATA BIOMOL, 'Online! 15 March 1997 (1997-03-15), XP002214385 Retrieved from the Internet: <URL:http://www.biomol.com/store/deptProducts.asp?dept%5Fid=107013&pf%5Fid=SA%2D317&Qsearch=&anchor=#SA-317> 'retrieved on 2002-09-13! the whole document	10,13
X	CHENG X ET AL: "Phosphorylation and activation of cAMP-dependent protein kinase by phosphoinositide-dependent protein kinase." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA. UNITED STATES 18 AUG 1998, vol. 95, no. 17, 18 August 1998 (1998-08-18), pages 9849-9854, XP001094194 ISSN: 0027-8424 *page 9850, l. col., bottom line - r. col., paragraph 1*	1-7,9
X	MAHONEY CHARLES W ET AL: "High-throughput nonradioisotopic detection of picomole levels of phosphothreonine and phosphoserine containing peptides via biotinylation and enzyme-linked immunosorbent assay." ANALYTICAL BIOCHEMISTRY, vol. 268, no. 2, 15 March 1999 (1999-03-15), pages 371-376, XP001095804 ISSN: 0003-2697 page 375 -page 376	1-7

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	ARIMURA NARIKO ET AL: "Phosphorylation of collapsin response mediator protein-2 by Rho-kinase. Evidence for two separate signaling pathways for growth cone collapse." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, no. 31, 4 August 2000 (2000-08-04), pages 23973-23980, XP001094193 ISSN: 0021-9258 *abstract; page 23974, r. col. "Production of Site- and Phosphorylation State-specific Antibody ..." ---	9
X	KIROYCHEVA M ET AL: "Mitogen-activated protein kinase phosphorylation in kidneys of beta(s) sickle cell mice." JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY: JASN. UNITED STATES JUN 2000, vol. 11, no. 6, June 2000 (2000-06), pages 1026-1032, XP002214386 ISSN: 1046-6673 *page 1027 - 1028, sections headed "Western Blot" and "Phosphorylation of p38 MAP Kinase and Effect of MEG"* ---	9,10
X	GATTI ANDREA ET AL: "Phosphorylation of human p53 on Thr-55." BIOCHEMISTRY, vol. 39, no. 32, 15 August 2000 (2000-08-15), pages 9837-9842, XP002214387 ISSN: 0006-2960 antibody specific for Thr-55 of p53; immunoassay of phosphokinase activity on the protein; pan anti-p53 antibody also used ---	1-7,9, 14-20
X	ICELY P L ET AL: "TIK A NOVEL SERINE-THREONINE KINASE IS RECOGNIZED BY ANTIBODIES DIRECTED AGAINST PHOSPHOTYROSINE" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 24, 1991, pages 16073-16077, XP001097726 ISSN: 0021-9258 *abstract; page 16076, r. col., bottom paragraph* ---	10

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	PALMER HELEN J ET AL: "Age-dependent decline in mitogenic stimulation of hepatocytes. Reduced association between Shc and the epidermal growth factor receptor is coupled to decreased activation of Raf and extracellular signal-regulated kinases." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 16, 16 April 1999 (1999-04-16), pages 11424-11430, XP001097727 ISSN: 0021-9258 abstract ---	10,13
X	ROKKA ANNE ET AL: "Dephosphorylation of photosystem II reaction center proteins in plant photosynthetic membranes as an immediate response to abrupt elevation of temperature." PLANT PHYSIOLOGY (ROCKVILLE), vol. 123, no. 4, August 2000 (2000-08), pages 1525-1535, XP001095802 ISSN: 0032-0889 page 1533, left-hand column, paragraph 2 ---	9
X	WO 99 29894 A (EPPS DENNIS E ;UPJOHN CO (US); MARSCHKE CHARLES K (US)) 17 June 1999 (1999-06-17) claims 1-35 -----	1-7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 01/30186

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [X] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

International Application No. PCT/US 01 /30186

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 8,11,12

   Antibody raised to a polypeptide immunogen having a phosphorylated serine, in particular antibodies specific to sequence IDs 1 and 2 derived from tau protein

2. Claims: 9,14

   Antibodies raised to a polypeptide immunogen having a phosphorylated threonine, in particular threonine 821

3. Claims: 10,13

   Antibodies raised to a polypeptide immunogen having a phosphorylated tyrosine, in particular tyrosine 1173 or 845 of EGFR

4. Claims: 1-7,15-20

   Methods for measuring phosphokinase activity relying on an antibody specific to a phosphorylated phosphorylation site on a protein; kits for such methods.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 01/30186

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9924473 A	20-05-1999	DE 19749091 C1	29-07-1999
		AU 1871299 A	31-05-1999
		BR 9813988 A	26-09-2000
		CA 2308604 A1	20-05-1999
		CN 1279691 T	10-01-2001
		WO 9924473 A1	20-05-1999
		EP 1042368 A1	11-10-2000
		HU 0004099 A2	28-03-2001
		JP 2001522865 T	20-11-2001
		PL 340465 A1	12-02-2001
		TR 200001265 T2	21-09-2000
EP 0544942 A	09-06-1993	EP 0544942 A1	09-06-1993
		AT 185599 T	15-10-1999
		AU 707736 B2	15-07-1999
		AU 2865697 A	04-12-1997
		AU 681071 B2	21-08-1997
		AU 3256093 A	28-06-1993
		CA 2125298 A1	10-06-1993
		DE 69230148 D1	18-11-1999
		DE 69230148 T2	04-05-2000
		DK 618968 T3	10-04-2000
		WO 9311231 A1	10-06-1993
		EP 0618968 A1	12-10-1994
		EP 0911398 A2	28-04-1999
		EP 0911390 A2	28-04-1999
		EP 0909814 A2	21-04-1999
		ES 2136654 T3	01-12-1999
		JP 2000026498 A	25-01-2000
		JP 2000037188 A	08-02-2000
		JP 2000032983 A	02-02-2000
		JP 7507044 T	03-08-1995
		US 6200768 B1	13-03-2001
WO 9604309 A	15-02-1996	AU 710952 B2	30-09-1999
		AU 3223495 A	04-03-1996
		CA 2195672 A1	15-02-1996
		WO 9604309 A1	15-02-1996
		EP 0772634 A1	14-05-1997
		JP 10506381 T	23-06-1998
		US 6121003 A	19-09-2000
WO 9929894 A	17-06-1999	AU 738194 B2	13-09-2001
		AU 1703499 A	28-06-1999
		CA 2306686 A1	17-06-1999
		EP 1036192 A1	20-09-2000
		JP 2001526380 T	18-12-2001
		WO 9929894 A1	17-06-1999
		US 6203994 B1	20-03-2001
		US 2001044117 A1	22-11-2001